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14. ABSTRACT

Members of the BCL-2 family of cell death regulators play critical roles in the progression of androgen-independent, metastatic prostate cancer. Despite years of research, the molecular mechanisms underlying the effects of these proteins remain unclear. In previous studies we demonstrated that BCL-2 family proteins regulate a crucial step in the apoptotic pathway (cytochrome c release) by regulating endoplasmic reticular and mitochondrial calcium fluxes. In this project we are studying these effects in more detail, focusing on the possibility that the so-called "BH3 only" proteins and Bax directly promote endoplasmic reticular calcium release. To this end, we are (1) Defining the effects of mitochondrial calcium uptake on cytochrome c mobilization and release; (2)Determining the effects of BH3 only members of the BCL-2 family on intracellular calcium fluxes; and (3) Identifying possible direct effects of Bax and Bak on ER calcium fluxes. With this information in hand, we expect that we will be able to define therapeutic strategies that directly target the cell death resistance mechanisms that appear to limit the effects of currently available therapies.

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Table of Contents

INTRODUCTION

Prostate cancer remains the most common malignancy in American men and is the second leading cause of cancer-related death (1). Advances in early detection have led to better surgical control of the disease, but less progress has been made in the treatment of metastatic cancer. Androgen ablation remains the therapy of choice for patients with metastatic prostate cancer, and almost all patients initially receive benefit from this approach. However, almost all patients ultimately relapse with androgen-independent cancer, and the treatment options for these patients are limited (2).

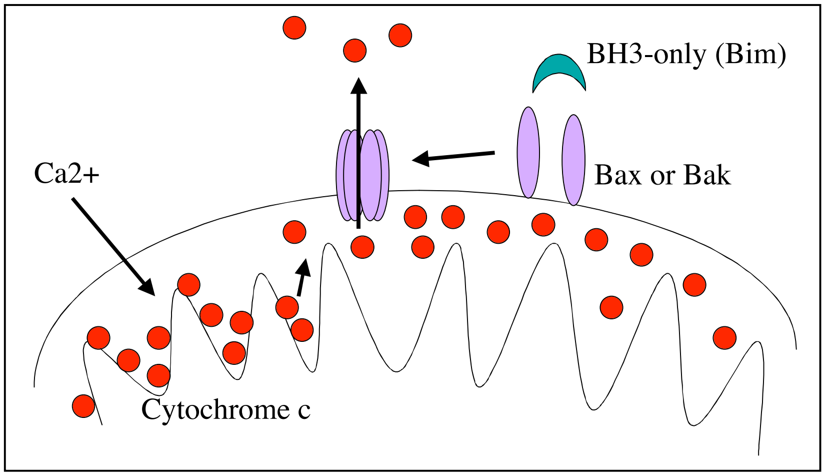
Investigation into the molecular mechanisms that mediate the effects of androgen ablation has established that apoptosis plays a central role, and defects in the control of apoptosis in androgen-independent tumors not only undermine androgen ablation therapy but also produce cross-resistance to other therapeutic modalities (3). Of particular importance are members of the BCL-2 family of cell death regulators, which are known to play evolutionarily conserved roles in the regulation of cell death in organisms ranging from the nematode *Caenorhabditis elegans* to humans (4). The family is subdivided into 3 major categories based on their functions in cell death regulation (4, 5). One subfamily (exemplified by BCL-2 itself and BCL-X_L) consists of cell death inhibitors and function to prevent the rate-limiting step in most examples of apoptosis (release of cytochrome c from mitochondria). The second (exemplified by Bax and Bak) are highly homologous to BCL-2 but function to directly promote cytochrome c release, possibly by forming transmembrane pores in the outer mitochondrial membrane. These proteins have been termed the "multidomain" proapoptotic BCL-2 family members, and expression of at least one of them appears to be required for initiation of apoptotic cell death. Finally, a third subfamily shares structural homology with the other two only within a circumscribed region (the so-called "BH3" domain), and these proteins appear to function either as inhibitors of the anti-apoptotic members of the family or as activators of Bax or Bak (6, 7). Overall, it appears that BH3 proteins function to directly or indirectly promote the tetramerization of Bax and/or Bak, which generates a pore that is large enough to accommodate cytochrome c release (8). By virtue of their abilities to directly bind (and presumably neutralize) the BH3-only and multidomain family members, the effects of BCL-2 and BCL-X_L can be attributed to their functions as pore inhibitors.

Although the pore formation model is relatively simple and probably accounts for an important aspect of BCL-2 family protein functions, strong evidence is now available that indicates that pore formation is not enough to mediate cytochrome c release. Specifically, permeabilization of the outer mitochondrial membrane only releases approximately 15% of the total pool of cytochrome c, whereas up to 90% of the pool is released in cells dying by apoptosis (9, 10). Thus, cytochrome c release appears to commence via a two-step process. In the first step, a tightly bound pool of cytochrome c is mobilized, and in the second Bax/Bak pores allow for this mobilized cytochrome c to escape into the cytosol. It is possible that BH3-only proteins might use different domains to accomplish both effects, as has been shown in the case of a truncated form of the BH3-only protein, Bid (9). However, other work indicates that disruption of the electrostatic interactions between cytochrome c and the charged mitochondrial membrane lipid, cardiolipin, can also lead to cytochrome c mobilization (11).

BCL-2 is overexpressed in androgen-independent prostate cancers (12, 13), whereas expression of Bax may be reduced (13). Thus, understanding the molecular mechanisms underlying their effects on apoptosis should help to identify new therapeutic strategies to overcome the cell death resistance observed in androgen-independent disease. Furthermore, over the past year Abbott reported the identification and molecular characterization of a novel small molecule inhibitor of BCL-2 that could prove to be a potent apoptosis-sensitizing agent in a subset of human prostate tumors once the molecular signature of BCL-2-dependent tumors has been defined (14). The overall goal of the research outlined in this project is to gain a better understanding of the effects of BCL-2 and Bax in prostate cancer cells. Our hypothesis is that an important component of the effects of these proteins is the regulation of intracellular calcium fluxes. More specifically, we suggest that proapoptotic members of the BCL-2 family promote release of calcium from its natural intracellular storage site (the endoplasmic reticulum),

leading to increases in mitochondrial calcium that function to mobilize cytochrome c. Our model is summarized in **Figure 1.**

Figure 1: Two-step model for Ca2+-dependent cytochrome c release. Mitochondrial Ca2+ uptake "loosens" interactions between cytochrome c and the inner mitochondrial membrane, allowing for release via tetrameric Bax and/or Bak channels in the outer membrane. The effects of Ca2+ may be due to disruption of interactions between cytochrome c and cardiolipin or to more global changes in mitochondrial structure.



BODY

Summary of progress during Year 1

Our overall approach was to overexpress wild-type or organelle-targeted forms of BCL-2 in PC-3 cells to investigate the importance of ER versus mitochondrial localization on ER Ca^{2+} release, mitochondrial Ca^{2+} uptake, and downstream consequences of these Ca^{2+} fluxes (cytochrome c release, caspase activation, DNA fragmentation). As outlined in the narrative of the progress report for Year 1, the results of these studies appeared to confirm our hypothesis that ER localization of BCL-2 was sufficient to block mitochondrial Ca^{2+} uptake, implying that BCL-2 might exert direct effects on the ER Ca^{2+} pool. <u>However, we developed several major concerns with the approach and have adopted alternative strategies to address these questions in Year 2</u>. First, as discussed in the Progress Report for Year 1, even though the "stable" transfectants we generated arose as single-cell colonies after selection in high concentrations of antibiotic, analysis of BCL-2 protein expression by immunofluorescence staining and confocal microscopy demonstrated that up to 50% of cells in newly-expanded populations of cells had already lost high level BCL-2 expression (depending on the construct introduced), and the remaining BCL-2-positive cells quickly lost BCL-2 expression over the next few weeks of culture. The cells tended to retain expression of the wild-type and ER-targeted forms much better than they did the mitochondrial protein, in part because it appeared that overexpression of mitochondrial BCL-2 was toxic. <u>**In Year 2 we discovered that these problems were not limited to the PC-3 cells but were also observed (albeit to a lesser extent) in LNCaP-derived cells as well.**</u> Furthermore, parallel studies of protein localization by immunoblotting in isolated organelle fractions indicated that protein targeting to specific organelles was imperfect, and one of our collaborators (Dr. Ray Meyn, Department of Experimental Radiation Oncology) corroborated our concerns. Given that the targeted proteins were expressed at much higher levels than the endogenous protein, we could not conclude that the cell death inhibition observed with the ER-targeted proteins was due to direct effects on the ER (as opposed to effects of the pool of the ER-targeted protein that had "leaked" out into the mitochondria or nucleus).

We also performed additional experiments in the beginning of Year 2 that further undermined our enthusiasm for overexpression studies. In these experiments we stably transfected cells with a truncated form of BCL-2 lacking the C-terminal membrane-anchoring domain, which in theory should result in cytoplasmic localization and dramatically reduced effects on cell death. However, the transfectants displayed mostly <u>**nuclear**</u> BCL-2 localization, and they were almost as resistant to thapsigargin- and staurosporine-induced apoptosis as were cells expressing the wild-type or ER-targeted forms of BCL-2. Together with the problems cited above, these findings prompted us to shelve the targeted BCL-2 constructs.

We now suspect that overexpressed BCL-2 can act as an inhibitor of apoptosis wherever it is localized, as long as the cell can tolerate the level of BCL-2 overexpression. (The mitochondrial BCL-2 construct we used appears to be toxic, consistent with the findings of Distelhorst's group (15).) BCL-2 can bind to and neutralize the pro-apoptotic effects of most BH3-only proteins, Bax, and Bak, and it does not necessarily have to be present on the mitochondrial outer membrane to do so. Our conclusions are consistent with those of others, who have shown that ER-localized BCL-2 prevents Bax from translocating to mitochondria (16). This experience has undermined our overall enthusiasm for using overexpression systems in whole cells to study cell biology. Our concerns are shared by many other cell biologists who would rather see functional information obtained from cells expressing more physiological protein levels.

Progress in the second year of funding:

Objective 1: *Define the effects of mitochondrial calcium uptake on cytochrome c mobilization and release.* These studies are "on hold" pending the outcome of the experiments described below. We want to identify all of the relevant players before we reconstitute the system in isolated organelles. Specifically, we wish to determine whether or not Bim and/or other proapoptotic BCL-2 family members are required for the ER Ca^{2+} pool depletion we observe in whole cells. Once we have this information in hand we can attempt to reproduce the whole cell observations in isolated microsomes obtained from prostate cancer cell lines and/or mouse liver (our back-up system).

Objective 2: *Determine the effects of BH3-only members of the BCL-2 family on intracellular calcium fluxes.* Task 1: We defined the patterns of BH3-only protein expression during Year 1.

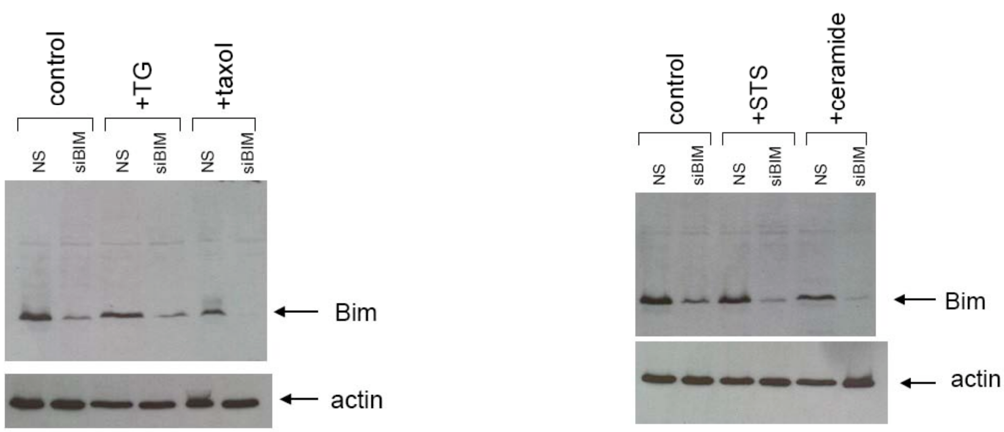
Tasks 2-4: These experiments were completed in PC-3 cells during Year 1 and were extended to LNCaP-Pro5 cells during Year 2.

Task 5: Most of our effort in Year 2 was dedicated to this sub-Aim. In direct response to the concerns raised above, we have shifted our focus away from overexpression studies to using siRNA technology to define the critical regulators of Ca^{2+} fluxes and apoptosis in whole prostate cancer cells, starting with the BH3-only protein Bim and the multi-domain protein, Bax. In the first phase of this work we characterized the effects of silencing Bim on endogenous endonuclease activation in LNCaP-Pro5 and PC-3 cells exposed to 4 major inducers of cell death: staurosporine, thapsigargin, ceramide, and paclitaxel. Our previous studies had demonstrated that staurosporine induces ER Ca^{2+} release in PC-3 cells and thapsigargin promotes ER Ca^{2+} release directly. Ceramide was reported to induce ER Ca^{2+} release by Korsmeyer's group in lymphoid cells (17), and taxanes are thought to induce apoptosis via a Bim-dependent mechanism (18); whether or not they also mobilize ER Ca^{2+} has not been established. (Taxanes are also components of frontline adjuvant therapy for androgen-independent prostate cancer in patients at our institution.)

In a first series of experiments we determined the kinetics and concentration-dependent effects of each of the aforementioned agents on apoptosis in PC-3 and LNCaP-Pro5 cells by propidium iodide staining and FACS analysis (PI/FACS). The results demonstrated that significant increases in apoptosis were observed in both cell lines by 24 h with maximal levels reached by 48 h, consistent with our previous experience (19, 20). We then compared the effects of several different strategies to deliver Bim siRNA transiently into PC-3 and LNCaP-Pro5 cells and ultimately selected a commercial liposome preparation (siImporter, from Upstate Biotechnology, Inc) that appeared to work more efficiently than other approached in both of the cell lines; representative results obtained by this strategy are displayed in **Figure 2** (LNCaP-Pro5 data are shown).

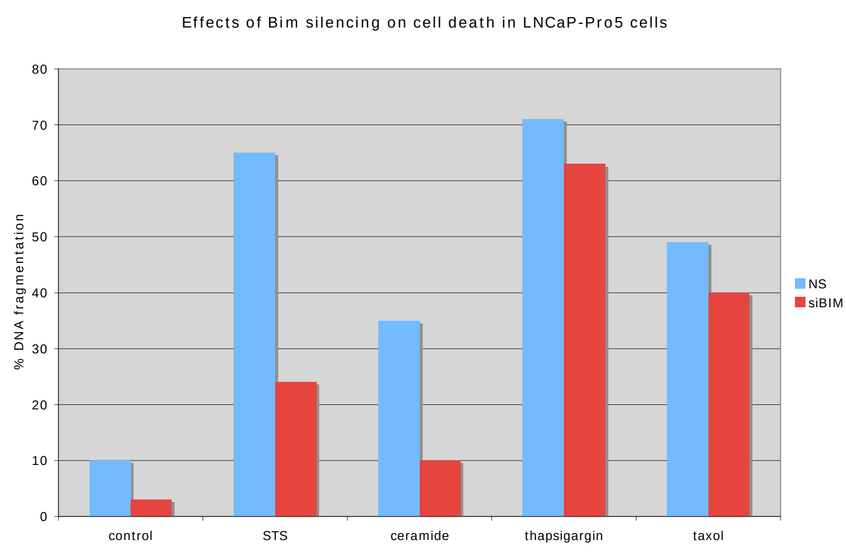
Once we had obtained optimal Bim knockdown we investigated the effects of Bim knockdown on DNA fragmentation induced by the 4 agents described above. Consistent with our hypothesis, the two agents that had previously been shown to trigger release of ER Ca^{2+} (staurosporine and ceramide) induced apoptosis via a Bim-

dependent mechanism (**Fig. 3**). Thus, Bim could in theory direct Bax and/or Bak activation within the ER, leading to ER Ca^{2+} release and subsequent mitochondrial Ca^{2+} uptake, which we plan to test over the next two months. In contrast, neither thapsigargin nor docetaxel induced apoptosis via a Bim-dependent mechanism (**Fig. 3**). We were not necessarily surprised to see that thapsigargin kills via a Bim-independent mechanism, since recent work has demonstrated that it induces apoptosis by triggering endoplasmic reticular (ER) stress (21, 22) and its direct effects on the ER Ca^{2+} ATPase would account for ER Ca^{2+} release independent of any effects of Bim and/or Bax. However, the results we obtained with docetaxel were surprising in light of the fact that Bim associates with microtubules (23), taxanes kill cells via microtubule stabilization, and previous work in other models implicated Bim in taxane-induced cell death (18). It is likely that the discrepancy lies in the use of different experimental model systems, and the results support the contention that findings obtained in other cellular model systems are not necessarily relevant to human prostate cancer. We are currently studying the effects of silencing Bax and/or Bak on cell death induced by these agents.



<u>Figure 2.</u> Effects of Bim silencing on Bim expression in LNCaP-Pro5 cells. Cells were transiently transfected with an siRNA construct specific for Bim or an off-target control. After 48 h, cells were exposed to optimal concentrations of thapsigargin (TG), docetaxel (taxol), staurosporine (STS), or ceramide for an additional 24 h, and Bim expression was quantified by immunoblotting. Results are representative of those obtained in at least 3 independent experiments. Note that Bim levels were decreased by over 80% under all conditions.

<u>Figure 3.</u> Effects of Bim silencing on apoptosis. LNCaP-Pro5 cells were transiently transfected with an siRNA construct specific for Bim or an off-target control for 48 h. Cells were subsequently exposed to 0.5 μM staurosporine (STS), 10 μM ceramide, 1 μM thapsigargin, or 10 μg/ml docetaxel for 24 h, and DNA fragmentation was measured by PI/FACS. Results are representative of at least 3 independent replicates.



We also attempted to use GFP-Bax constructs (from Richard Youle, NIH) to monitor Bax localization in LNCaP-Pro5 cells transiently transfected with these constructs. (We had previously tested the constructs in PC-3 cells.) Previous studies indicate that Bax localizes to the cytosol in resting cells and translocates to mitochondria during cell death (24). Furthermore, we have shown that staurosporine also induces translocation of Bax to the ER (20), a result that has since been confirmed by others (25). Thus, in theory, Bim could activate Bax in the ER, leading to the formation of transmembrane pores that might accommodate ER Ca^{2+} release. Although we obtained good expression of the fusion proteins, they displayed constitutive localization to mitochondria and triggered apoptosis independently of any other exogenous signal. Therefore, and consistent with the conclusions presented above, we plan to monitor endogenous Bax localization by immunofluorescence staining and confocal microscopy (in fixed cells) and by subcellular fractionation rather than by overexpression, since overexpression appears to bypass the need for an apoptotic stimulus to trigger Bax translocation to mitochondria.

<u>Objective 3:</u> *Identify the effects of Bax and Bak on ER calcium fluxes.*

Task 1: The planned studies with isolated organelles are "on hold" until we have determined whether or not Bim and/or other BH3-only proteins participate in ER Ca2+ release in whole cells.

Tasks 2 and 3: As discussed in the narrative description of the progress made during Year 1, our concerns about imperfect targeting of BCL-2 itself caused us to change our plans with regard to generating targeted forms of Bax and Bak. Instead, we will use immunofluorescence staining and confocal microscopy to first confirm our previous observation that the proteins translocate to the ER in response to Ca^{2+}-mobilizing stimuli (i.e., staurosporine and ceramide), and we will employ siRNA-mediated gene silencing to determine whether or not translocation requires BH3-only protein(s) (Bim remains our best candidate). Then we will focus on the isolated organelle studies to determine whether or not the pro-apoptotic protein(s) implicated in ER Ca^{2+} release in whole cells will promote ER Ca^{2+} pool depletion (using $^{45}Ca^{2+}$ uptake as an assay) in vitro.

KEY RESEARCH ACCOMPLISHMENTS

- Identified strategies to reproducibly and effectively silence gene expression in LNCaP and PC-3 cells
- Determined that Bim is required for cell death induced by two agents that promote ER Ca^{2+} release (staurosporine and ceramide) but not for cell death induced by thapsigargin or docetaxel
- Determined that organelle-targeted forms of BCL-2 are "leaky" and that protein overexpression is reduced with passage in culture
- Determined that levels of GFP-Bax overexpression that allow for ready visualization of protein localization in whole cells leads to mitochondrial accumulation of the protein and increased spontaneous cell death

REPORTABLE OUTCOMES

1. Developed reliable strategies to silence Bim expression in PC-3 and LNCaP-Pro5 cells.
2. Used our optimized siRNA methods to knock down cdk1/cdc2 expression and further revise a manuscript entitled, "The proteasome inhibitor bortezomib interferes with docetaxel-induced apoptosis in human LNCaP-Pro5 prostate cancer cells," S. Canfield et al, *Molecular Cancer Therapeutics*. (Revised manuscript resubmitted.)
3. Used our confocal microscopy and organelle fractionation methods to determine the effects of hypoxia, cobalt chloride, and bortezomib on nuclear localization of the angiogenesis-associated transcription factor and bortezomib target Hif-1α in LNCaP-Pro5 cells (manuscript in preparation).

CONCLUSIONS

1. Bim is required for apoptosis induced by staurosporine and ceramide.
2. Bim is not required for apoptosis induced by thapsigargin or docetaxel.

3. Bim's lack of involvement in docetaxel-induced apoptosis strongly suggests that BCL-2 family proteins regulate apoptosis differently in human prostate cancer cells as compared to other human cell types.
4. Stable or transient overexpression of either pro- or anti-apoptotic proteins perturbs cell physiology in ways that makes their effects difficult to interpret.
5. Organelle-targeted forms of BCL-2 display significant "leakiness" and are as a consequence promiscuous inhibitors of cell death.

REFERENCES

1. Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J., and Thun, M. J. Cancer statistics, 2005. CA Cancer J Clin, *55:* 10-30, 2005.
2. Assikis, V. J. and Simons, J. W. Novel therapeutic strategies for androgen-independent prostate cancer: an update. Semin Oncol, *31:* 26-32, 2004.
3. Bruckheimer, E. M., Gjertsen, B. T., and McDonnell, T. J. Implications of cell death regulation in the pathogenesis and treatment of prostate cancer. Semin Oncol, *26:* 382-398, 1999.
4. Danial, N. N. and Korsmeyer, S. J. Cell death: critical control points. Cell, *116:* 205-219, 2004.
5. Chao, D. T. and Korsmeyer, S. J. BCL-2 family: regulators of cell death. Annu Rev Immunol, *16:* 395-419, 1998.
6. Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell, *2:* 183-192, 2002.
7. Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. BH3 Domains of BH3-Only Proteins Differentially Regulate Bax-Mediated Mitochondrial Membrane Permeabilization Both Directly and Indirectly. Mol Cell, *17:* 525-535, 2005.
8. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev, *14:* 2060-2071, 2000.
9. Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S. A., Mannella, C. A., and Korsmeyer, S. J. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell, *2:* 55-67, 2002.
10. Scorrano, L. and Korsmeyer, S. J. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun, *304:* 437-444, 2003.
11. Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci U S A, *99:* 1259-1263, 2002.
12. McDonnell, T. J., Troncoso, P., Brisbay, S. M., Logothetis, C., Chung, L. W., Hsieh, J. T., Tu, S. M., and Campbell, M. L. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res, *52:* 6940-6944, 1992.
13. McConkey, D. J., Greene, G., and Pettaway, C. A. Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. Cancer Res, *56:* 5594-5599, 1996.
14. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature, *435:* 677-681, 2005.
15. Wang, N. S., Unkila, M. T., Reineks, E. Z., and Distelhorst, C. W. Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. J Biol Chem, *276:* 44117-44128, 2001.

16. Thomenius, M. J., Wang, N. S., Reineks, E. Z., Wang, Z., and Distelhorst, C. W. Bcl-2 on the endoplasmic reticulum regulates Bax activity by binding to BH3-only proteins. J Biol Chem, *278:* 6243-6250, 2003.
17. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science, *300:* 135-139, 2003.
18. Tan, T. T., Degenhardt, K., Nelson, D. A., Beaudoin, B., Nieves-Neira, W., Bouillet, P., Villunger, A., Adams, J. M., and White, E. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell, *7:* 227-238, 2005.
19. Williams, S., Pettaway, C., Song, R., Papandreou, C., Logothetis, C., and McConkey, D. J. Differential effects of the proteasome inhibitor bortezomib on apoptosis and angiogenesis in human prostate tumor xenografts. Mol Cancer Ther, *2:* 835-843, 2003.
20. Nutt, L. K., Chandra, J., Pataer, A., Fang, B., Roth, J. A., Swisher, S. G., O'Neil, R. G., and McConkey, D. J. Bax-mediated Ca2+ mobilization promotes cytochrome c release during apoptosis. J Biol Chem, *277:* 20301-20308, 2002.
21. Nawrocki, S. T., Carew, J. S., Pino, M. S., Highshaw, R. A., Dunner, K., Jr., Huang, P., Abbruzzese, J. L., and McConkey, D. J. Bortezomib sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis. Cancer Res, *65:* 11658-11666, 2005.
22. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature, *403:* 98-103, 2000.
23. Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Kontgen, F., Adams, J. M., and Strasser, A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science, *286:* 1735-1738, 1999.
24. Hsu, Y. T., Wolter, K. G., and Youle, R. J. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A, *94:* 3668-3672, 1997.
25. Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol, *162:* 59-69, 2003.

Bortezomib Abolishes Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Resistance via a p21-Dependent Mechanism in Human Bladder and Prostate Cancer Cells

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of cytokines that induces apoptosis in some tumor cells but not in normal cells. Unfortunately, many human cancer cell lines are refractory to TRAIL-induced cell death, and the molecular mechanisms underlying resistance are unclear. Here we report that TRAIL resistance was reversed in human bladder and prostate cancer cell lines by the proteasome inhibitor bortezomib (PS-341, Velcade). Synergistic induction of apoptosis occurred within 4 to 6 hours in cells treated with TRAIL plus bortezomib and was associated with accumulation of p21$^{WAF-1/Cip-1}$ (p21) and inhibition of cyclin-dependent kinase (cdk) activity. Roscovitine, a specific cdk1/2 inhibitor, also sensitized cells to TRAIL. Silencing p21 expression reduced levels of DNA fragmentation by 50% in cells treated with bortezomib and TRAIL, confirming that p21 was required for the response. Analysis of the TRAIL pathway revealed that caspase-8 processing was enhanced in a p21-dependent fashion in cells exposed to TRAIL and bortezomib as compared with cells treated with TRAIL alone. Thus, all downstream components of the pathway (Bid cleavage, cytochrome *c* release, and caspase-3 activation) were amplified. These data strongly suggest that p21-mediated cdk inhibition promotes TRAIL sensitivity via caspase-8 activation and that TRAIL and bortezomib should be combined in appropriate *in vivo* models as a possible approach to solid tumor therapy. (Cancer Res 2005; 65(11): 4902-8)

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a homotrimeric cytokine that induces cell death in a variety of different cancer cell types but not in normal cells (1–3). TRAIL promotes apoptosis via binding to two surface receptors (DR4 and DR5) that contain homologous death domains within their cytoplasmic tails, resulting in receptor trimerization and recruitment of the cytosolic death domain–containing protein, Fas-associated death domain (FADD; refs. 4–7). This stimulated conformation of the TRAIL receptor, known as the death-inducing signaling complex (DISC; ref. 8), allows FADD to recruit and activate procaspase-8, which undergoes autocatalytic activation (9). Once fully activated, caspase-8 can either directly cleave and activate downstream effector caspases (3, 7) or it can stimulate a mitochondrial amplification loop by cleaving Bid, a BH3-only member of the Bcl-2 family (10–12). Studies in animal models indicate that systemic therapy with TRAIL is safe, and phase I clinical trials designed to evaluate TRAIL toxicity and antitumor efficacy are being opened this year (13). However, *in vitro* data show that up to 50% of tumor cell lines do not undergo apoptosis in response to TRAIL. Thus, understanding the molecular mechanisms underlying TRAIL resistance and identifying strategies to reverse it are high priorities for ongoing research.

The 26S proteasome is a multicatalytic enzyme expressed in the nucleus and cytoplasm of all eukaryotic cells that degrades proteins targeted by ubiquitin conjugation (14). The proteasome is responsible for maintaining homeostasis by controlling intracellular levels of cell cycle regulatory proteins (p21, p27, and p53), transcription factors, and certain tumor suppressor genes/oncogenes, making it an attractive therapeutic target in cancer (15–17). Bortezomib is a peptide boronate inhibitor of the proteasome that was developed as an anticancer agent several years ago and was the first such agent approved by the Food and Drug Administration for the treatment of a human cancer (multiple myeloma; ref. 18). It selectively binds to and inhibits the chymotryptic-like activity of the proteasome at nanomolar concentrations, and in the National Cancer Institute's 60 cancer cell line screen, bortezomib displayed a mean IC_{50} of 7 nmol/L with a unique spectrum of anticancer activity (19). Cellular responses depend on tumor type and range from cell cycle inhibition to apoptosis, and *in vivo* studies have shown that bortezomib inhibits the growth of a variety of different solid tumors without significant toxicity (19–23).

Here we report that TRAIL-resistant human prostate and bladder cancer cell lines can be rapidly sensitized to TRAIL-induced apoptosis by treating them with bortezomib. The molecular mechanisms underlying the effects of bortezomib involve p21 accumulation and enhanced activation of caspases 8 and 3.

Materials and Methods

Cell culture and reagents. The LNCaP-derived cell line, LNCaP-Pro5 (24), was generously provided by Dr. Curtis Pettaway (Department of Urology, University of Texas M.D. Anderson Cancer Center). The 253J B-V cells were derived from the 253J parental line by orthotopic "recycling" through the mouse bladder as described previously (25). The UM-UC3 cells were obtained from H. Barton Grossman (Department of Urology, University of Texas M.D. Anderson Cancer Center). Human PC-3 and DU-145 prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD). The prostate cancer cells were grown in RPMI

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1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies) and 1% MEM vitamin solution (Life Technologies), sodium pyruvate (Bio Whittaker, Rockland, ME), L-glutamine (Bio Whittaker), penicillin/streptomycin solution (Bio Whittaker), and nonessential amino acids (Life Technologies) under an atmosphere of 5% CO_2 in an incubator. The bladder cancer cells were cultured in MEM containing the same supplements. Bortezomib was kindly supplied by Millenium Pharmaceuticals (Cambridge, MA), and recombinant human TRAIL (rhTRAIL) was purchased from R&D Systems, Inc. (Minneapolis, MN).

Quantification of apoptosis by propidium iodide/fluorescence-activated cell sorting. Cells were treated with 10 ng/mL of rhTRAIL and/or 100 nmol/L bortezomib for the times indicated. Both growth and wash medium were saved and cells were harvested with trypsin. Supernatants were removed and pellets were resuspended in 400 μL of propidium iodide (PI) solution (50 μg/mL PI, 0.1% Triton X-100, and 0.1% sodium citrate in PBS). Samples were then incubated overnight at 4°C in the dark before analysis by flow cytometry. The cells with subdiploid DNA content were quantified to determine the percentage of cells containing apoptotic, fragmented DNA (26).

Quantitative analysis of phosphatidylserine exposure. Cells were treated with 10 ng/mL of rhTRAIL and/or 100 nmol/L bortezomib for the times indicated before harvest with trypsin. Exposure of phosphatidylserine was measured by Annexin V binding as described previously (27) using a commercial kit (Annexin V/PE Apoptosis Detection kit, BD Biosciences, San Diego, CA) according to manufacturer's protocol. Cell pellets were washed twice with cold PBS and resuspended in 1× binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$] at a concentration of 1×10^6 cells/mL. Aliquots of 100 μL were transferred to separate tubes and 5 μL of Annexin V/PE plus 5 μL of 7-AAD (7-amino-actinomycin D) were added to each. After vortexing, cells were incubated at room temperature for 15 minutes in the dark. Samples were diluted with 400 μL of 1× binding buffer, and surface Annexin V immunofluorescence was quantified immediately by flow cytometry.

Immunoblot analyses. Cells were lysed by incubation for 1h at 4°C in 100 μL of Triton lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and one Complete Mini Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN)]. Lysates were centrifuged for 5 minutes at 12,000 × *g* (4°C), and 20 μg of the postnuclear supernatants were mixed with equal volumes of 2× SDS-PAGE sample buffer (50 mmol/L Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol). Samples were then boiled, for 5 minutes at 100°C and resolved by 15% SDS-PAGE at 100 V for 90 minutes. Polypeptides were transferred to nitrocellulose membranes for 90 minutes at 100 V in a transfer buffer containing 39 mmol/L glycine, 48 mmol/l Tris, and 20% methanol. Membranes were blocked for 1 hour in 5% milk diluted in TBS containing 0.1% Tween 20 (TBS-T). Membranes were incubated overnight at 4°C with primary antibodies specific for caspase-8 (Cell Signaling Technology, Beverly, MA; 1:1,000 dilution), caspase-3, cytochrome *c*, p21, or p27 (PharMingen, San Diego, CA; 1:1,000 dilution), or Bid (R&D Biosystems, Minneapolis, MN; 1:1,000 dilution). Blots were washed 3× 5 minutes in TBS-T before incubation with secondary antibodies (horseradish peroxidase–conjugated sheep antimouse or donkey anti-rabbit antibody; Amersham Biosciences, Piscataway, NJ; 1:1,000 dilution) for 2 hours at 4°C. Blots were washed 3× 10 minutes in TBS-T and developed by enhanced chemiluminescence (Renaissance; New England Nuclear, Boston, MA).

Caspase-3 assay. Cells were treated with 100 nmol/L bortezomib and/or 10 ng/mL rhTRAIL for the times indicated and harvested with trypsin. Growth and wash medium were saved and cell pellets were washed once with PBS. Supernatants were removed and pellets were lysed with 200 μL cold lysis buffer [100 mmol/L HEPES (pH 7.4), 1% sucrose, 0.1% CHAPS, 1 mmol/L EDTA, 100 mmol/L DTT] containing a protease inhibitor cocktail ("Complete Mini" Protease Inhibitor Tablet, Boehringer, Indianapolis, IN). Cells were lysed at 4°C for 1 hour and centrifuged, and 800 μL of caspase buffer plus 2 μL of 20 mmol/L DEVD-AFC fluorogenic substrate (AFC 138, Enzyme Systems Products, Livermore, CA) was added to each supernatant. Samples were incubated for 1 hour at 37°C in the dark and diluted with 1 mL caspase buffer, and released AFC fluorescence was quantified using a Shimadzu spectrofluorimeter (Model RF-1501).

Immune complex cdk2 kinase assays. Cells were cultured to 60% confluency in 10-cm dishes and treated with various concentrations of bortezomib or roscovitine for 24 hours. Cells were then harvested with trypsin and lysed by rotating them for 1 hour at 4°C in 1 mL of the Triton X-100 lysis buffer described above. Lysates were cleared by centrifugation for 10 minutes at 12,000 × *g* (4°C). Supernatants containing 400 μg of protein were then incubated with an anti-cdk2 antibody for 2 hours followed by overnight incubation with 50 μL protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. The beads were then washed twice with lysis buffer and twice more with kinase buffer [25 mmol/L Tris (pH 7.2) and 10 mmol/L $MgCl_2$]. Immunoprecipitates were incubated with 1 μg histone H1, 150 μmol/L ATP, and 20 μCi [γ-^{32}P] ATP in 50 μL of kinase buffer for 15 minutes at 30°C. SDS sample buffer was used to terminate the reaction and the mixture was boiled for 5 minutes at 100°C. Finally, the mixture was loaded onto 12% SDS-PAGE gels and resolved at 100 V for 90 minutes. The gels were stained with Coomassie blue, destained, dried, and analyzed by autoradiography.

Small interfering RNA–mediated silencing of p21. Cells were grown to 60% confluency in 6-well plates and transfected with specific or nonspecific small interfering RNA (siRNA) constructs for 48 hours according to the manufacturer's protocols. The constructs used were the siRNA SMARTpool cdk-N-1A ($p21^{WAF-1/Cip-1}$) and cdk-N-1B ($p27^{Kip-1}$) (Upstate Cell Signaling Solutions, Lake Placid, NY) or the siRNA Nonspecific Control IV (Dharmacon RNA Technologies, Lafayette, CO), all at 200 nmol/L. Liposome-mediated transfection was accomplished with Oligofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) diluted 1:100 in serum-free MEM. Following silencing cells were treated with rhTRAIL (10 ng/mL) and bortezomib (100 nmol/L) for 8 hours and DNA fragmentation was quantified by PI/fluorescence-activated cell sorting (FACS). The efficiency of p21 or p27 silencing was verified in each experiment by immunoblotting.

Results

Effects of bortezomib on tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis. Previous studies have implicated the nuclear factor κB (NF-κB) pathway in the regulation of TRAIL resistance (28, 29). In preliminary experiments, we found that many human bladder and prostate cancer cell lines are refractory to TRAIL-induced apoptosis at baseline. Hypothesizing that NF-κB activation maintains the resistant phenotype in these cell lines, we treated them simultaneously with TRAIL plus bortezomib (a potent NF-κB antagonist; ref. 30) and measured DNA fragmentation by PI/FACS analysis 24 hours later. The results revealed a dramatic synergistic interaction between bortezomib and TRAIL in all of the cell lines (Fig. 1*A*). We confirmed these results using an independent measure of apoptosis (Annexin V staining) for detection of phosphatidylserine externalization (Fig. 1*B*). In contrast, a more selective inhibitor of NF-κB (the IKK antagonist PS-1145; ref. 31) had no effect on TRAIL-induced apoptosis (Fig. 1*C*), strongly suggesting that NF-κB inhibition did not account for the effects of bortezomib on TRAIL sensitivity.

In subsequent experiments, we characterized the effects of bortezomib on critical components of the TRAIL cell death pathway. Kinetic analyses showed that TRAIL sensitization occurred as early as 4 to 6 hours in the LNCaP Pro5 and 253JB-V cells (60.0 ± 8.56, $P < 0.001$ and 60.8 ± 10.5, $P < 0.001$, respectively; Fig. 2*A*). Immunoblotting studies showed that bortezomib had no

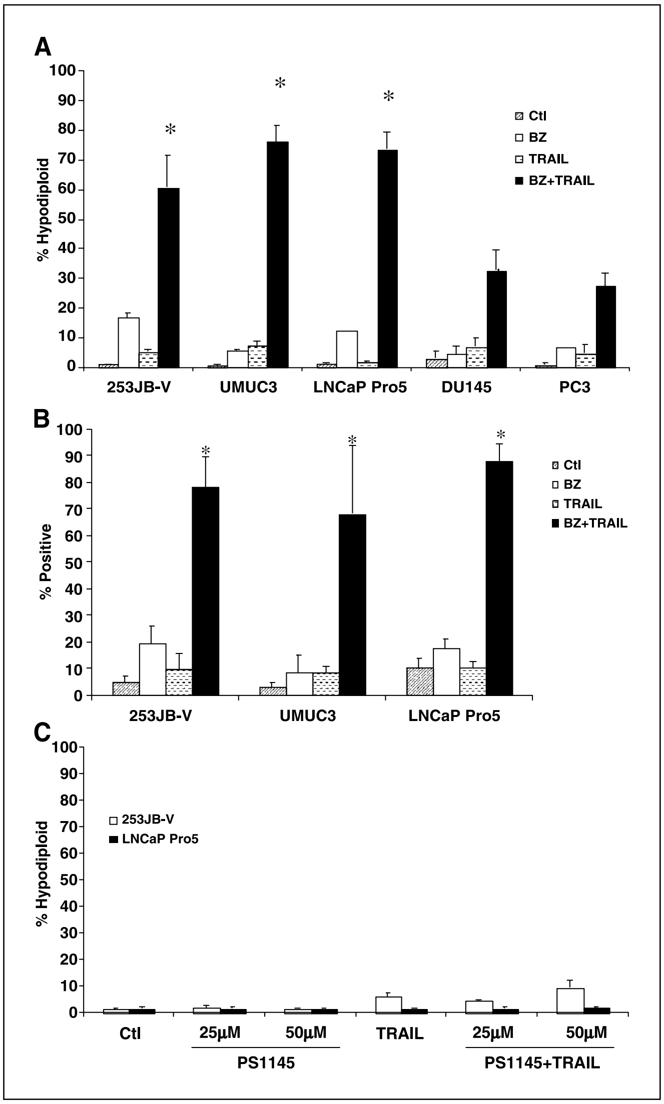

Figure 1. Effects of bortezomib on TRAIL-induced apoptosis. *A,* effects on DNA fragmentation. Cells were incubated in the absence or presence of 10 ng/mL rhTRAIL with or without 100 nmol/L bortezomib (*BZ*) for 24 hours and DNA fragmentation was quantified by PI/FACS as described in Materials and Methods. *Columns,* mean ($n = 3$); *bars,* ±SE. *, $P < 0.01$. *B,* effects on phosphatidylserine exposure. Cells were incubated for 24 hours as described above, and externalization of PS was quantified by immunofluorescence Annexin V staining and FACS analysis as described in Materials and Methods. *Columns,* mean ($n = 3$); *bars,* ±SE. *, $P < 0.01$. *C,* effects of the IKK inhibitor, PS-1145, on TRAIL-induced apoptosis. Cells were incubated for 24 hours in the absence or presence of 10 ng/mL rhTRAIL with or without 50 μmol/L PS-1145 and DNA fragmentation was measured by PI/FACS as described in Materials and Methods. *Columns,* mean ($n = 3$); *bars,* ±SE.

effect on proteolytic processing and activation of caspase-8, whereas incubation with TRAIL resulted in partial proteolytic processing of caspase-8 to form a 43- to 41-kDa intermediate by 8 hours (Fig. 2*B*). In cells treated with bortezomib plus TRAIL, this intermediate formed with much more rapid kinetics (<30 minutes), and it was accompanied by the formation of a smaller fragment (18 kDa) characteristic of the active, large subunit of caspase-8 (Fig. 2*B*). Cells treated with TRAIL plus bortezomib in the presence of a caspase-8–selective peptide antagonist, IETDfmk (10 μmol/L), displayed no DNA fragmentation above controls (data not shown), consistent with previous studies that showed that caspase-8 is required for TRAIL-induced cell death (32). Cells treated with TRAIL plus bortezomib also displayed enhanced cleavage of Bid and release of cytochrome *c* (Fig. 2*C*). Finally, exposure to either bortezomib or TRAIL alone had little effect on procaspase-3, whereas treatment with the combination promoted rapid proteolytic processing of procaspase-3 and its enzymatic activation (Fig. 2*D*). Together, these data show that bortezomib interacts with the TRAIL pathway at the level of caspase-8 to promote the initiation of mitochondrial events (cytochrome *c* release) that dramatically amplify caspase-3 activation. These effects probably account for the synergistic induction of DNA fragmentation and phosphatidylserine exposure observed in cells treated with the combination.

Role of p21 in bortezomib-induced tumor necrosis factor–related apoptosis-inducing ligand sensitization. Although treatment with bortezomib alone failed to induce significant increases in apoptosis in the tested cell lines, previous work from our laboratory showed that it blocks DNA synthesis at low nanomolar concentrations in bladder cancer cells irrespective of whether or not it induces cell death (33). The effects on DNA synthesis are associated with accumulation of cyclin-dependent kinase (cdk) inhibitors, p21 and p27, and inhibition of cdk2 and cdc2 activity (30, 33, 34). Furthermore, p21 accumulation is considered a marker for effective inhibition of the proteasome (35). Consistent with the previous studies, bortezomib induced a time-dependent accumulation of p21 in all of the TRAIL-resistant cells examined here (Fig. 3*A*; data not shown). Bortezomib also stimulated increases in p27 expression with similar kinetics (Fig. 4*B*; data not shown). Immune complex kinase assays confirmed that accumulation of p21 and p27 was associated with inhibition of cdk2 activity (Fig. 2*B*).

To determine whether or not cdk inhibition was sufficient to promote TRAIL sensitization, we examined the effects of the broad-spectrum cdk inhibitor, roscovitine, on TRAIL-induced apoptosis. Roscovitine had no effect on apoptosis at the concentration and time point studied in the 253J B-V cells but did induce DNA fragmentation in LNCaP-Pro5 cells (Fig. 3*C*). Combined treatment with roscovitine plus TRAIL resulted in synergistic induction of DNA fragmentation in both cell lines as measured by PI/FACS (Fig. 2*C*). Similar results were obtained with another, structurally unrelated cdk inhibitor (olomoucine) but not with an inactive structural analogue of the compound (*iso*-olomoucine; data not shown). Together, these results suggest that inhibition of cdk activation is sufficient to explain the effects of bortezomib on TRAIL sensitization. However, cdk inhibitors (i.e., flavopiridol) can also interfere with transcription (36, 37), and these off-target effects may contribute to the TRAIL sensitization observed in cells treated with roscovitine or olomoucine as well.

To more directly assess the involvement of p21 and p27 in bortzomib-mediated TRAIL sensitization, we compared the levels of DNA fragmentation observed in LNCaP-Pro5 cells exposed to a control siRNA construct with those observed in LNCaP-Pro5 cells exposed to siRNA specific for p21 or p27. Immunoblotting confirmed that silencing was efficient in cells exposed to either of the specific constructs but not in the controls (Fig. 4*A* and *B*). Levels of DNA fragmentation in the p21-silenced cells were significantly lower than those observed in controls (32% versus 66%, or a 50% reduction, $P < 0.001$), confirming that the bortezomib-induced accumulation of p21 contributed directly to TRAIL sensitization. Levels in the p27-silenced cells also seemed

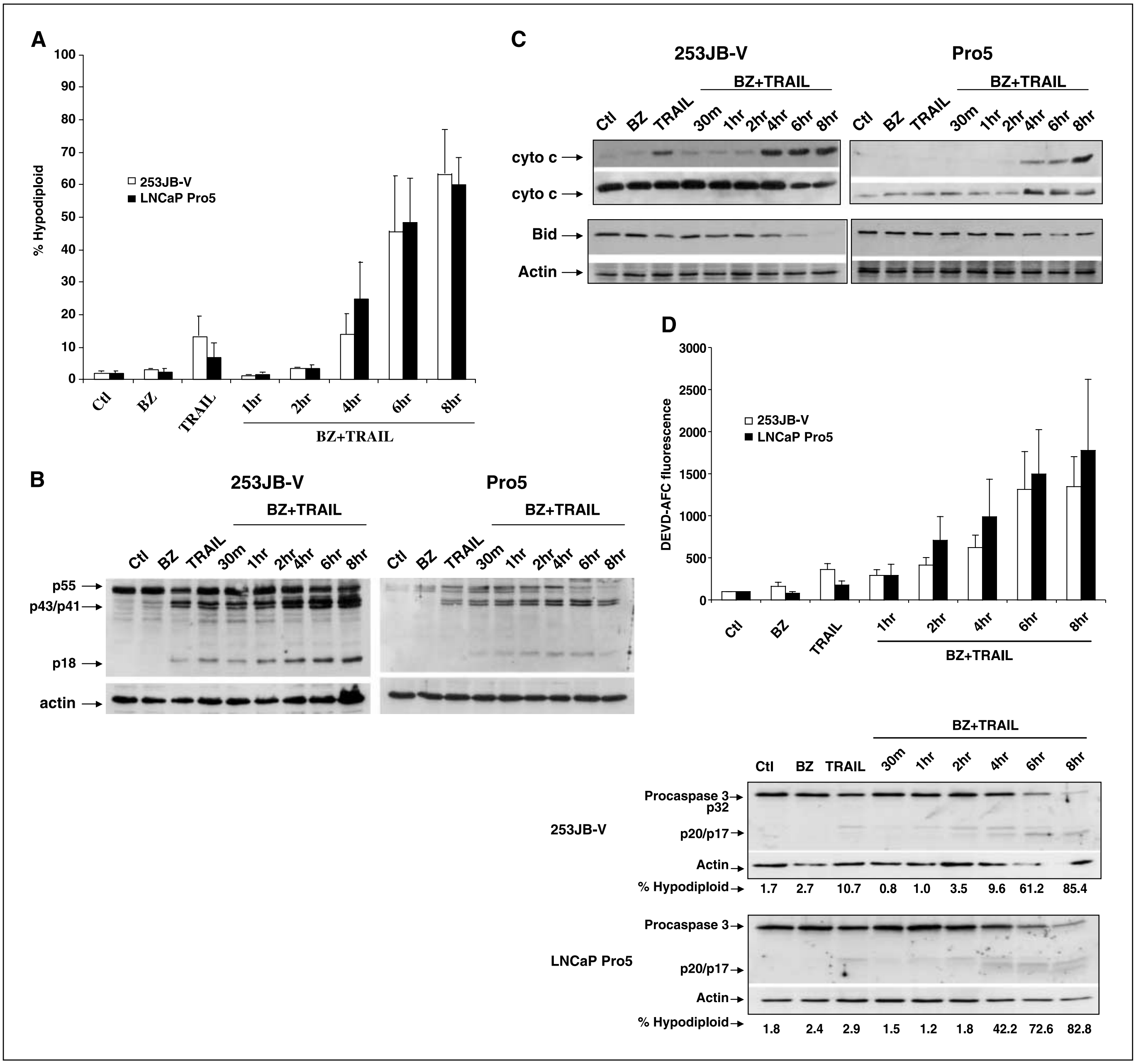

Figure 2. Characterization of the effects of bortezomib on sentinel steps within the TRAIL cell death pathway. *A,* kinetics of DNA fragmentation. 253J B-V or LNCaP-Pro5 cells were incubated in the absence or presence of 10 ng/mL TRAIL, 100 nmol/L bortezomib (*BZ*), or both for the times indicated and DNA fragmentation was measured by PI/FACS as described in Materials and Methods. *Columns,* mean ($n = 3$); *bars,* ±SE. *B,* effects on procaspase-8 processing. 253J B-V or LNCaP-Pro5 cells were incubated with TRAIL with or without bortezomib for the times indicated and proteolytic processing (activation) of procaspase-8 was analyzed by immunoblotting. Note that the appearance of the completely processed, active p18 caspase-8 fragment appears within 30 minutes in both cell lines. Representative of those obtained in three separate experiments. *C,* effects on cytochrome *c* release (*top*) and Bid cleavage (*bottom*). Cells were incubated with 10 ng/mL TRAIL with or without 100 nmol/L bortezomib for the times indicated. Cytochrome *c* release was measured in cytosolic extracts and the disappearance of the intact form of Bid was measured in total cell extracts by immunoblotting as described in Materials and Methods. In the cytochrome *c* experiment, the *top panels* display cytochrome *c* content of the cytosolic fractions, and the *bottom panels* display cytochrome *c* content of the membrane fractions. Note that the TRAIL plus bortezomib combination induces cytochrome *c* release by 4 hours in both cell types. Representative of those obtained in three separate experiments. *D,* effects on caspase-3 activation. 253J B-V or LNCaP-Pro5 cells were incubated with 10 ng/mL TRAIL in the absence or presence of 100 nmol/L bortezomib for the times indicated. Caspase-3 proteolytic activity was quantified in cytosolic extracts using a DEVD-AFC fluorigenic peptide as described in Materials and Methods (*top*). *Columns,* mean; *bars,* ±SD. Proteolytic processing of procaspase-3 was also measured in total cell extracts by immunoblotting. Percentages of hypodiploid cells were measured in parallel and are below each immunoblot. Note the appearance of active p20/p17 fragments by 4 hours in cells treated with TRAIL plus bortezomib. Representative of those obtained in three separate experiments.

consistently lower than in controls (Fig. 4*B*), but the effects did not reach statistical significance, and our attempts to simultaneously silence both p21 and p27 were unsuccessful. Silencing p21 inhibited procaspase-8 activation as measured by immunoblotting (Fig. 4*C*) or using a fluorigenic caspase-8 peptide substrate (data not shown), demonstrating that p21 acted at the level of procaspase-8 to promote cell death. Thus, processing of procaspase-3 was also reduced in cells depleted of p21 (Fig. 4*C*).

Discussion

Bortezomib and TRAIL are undergoing evaluation in clinical trials in a variety of different malignancies. Here we report that they can be combined to induce synergistic cell death in genitourinary cancer cells *in vitro* and *in vivo*. Characterization of the molecular mechanisms involved link the effects of bortezomib to increased caspase-8 activation, indicating that the drug affects TRAIL sensitivity at one of the earliest steps in the pathway. Cell death occurred with strikingly rapid kinetics (4-8 hours) as compared with responses to single or combined conventional chemotherapeutic agents, which in our hands require 24 to 48 hours in these cells. In fact, the kinetics of cell death observed here were more rapid than any we have observed in a solid tumor model exposed to any agent, including pharmacologic agents (staurosporine and thapsigargin) that are considered the most potent triggers of cell death.

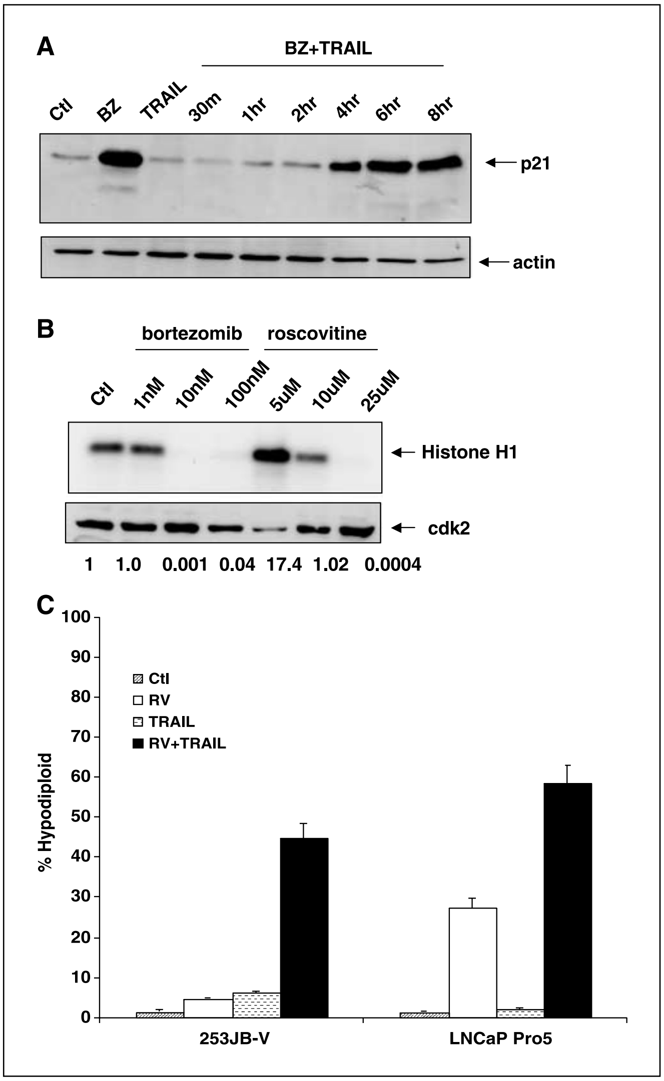

Figure 3. Effects of bortezomib on cdk activity. *A,* effects of bortezomib on p21 accumulation in LNCaP-Pro5 cells. Cells were incubated in the absence or presence of 100 nmol/L bortezomib, 10 ng/mL TRAIL, or both, and p21 expression was measured in total cell extracts by immunoblotting (*top*). Expression of actin was measured in parallel as a loading control. Representative of those obtained in three separate experiments. *B,* effects of bortezomib and roscovitine on cdk2 activity. LNCaP-Pro5 cells were incubated for 16 hours in the presence of the indicated concentrations of either agent and cdk2 kinase activity was measured in immunoprecipitates as described in Materials and Methods. Expression of cdk2 protein was quantified in parallel by immunoblotting and served as a loading control. Relative kinase activities were quantified in each condition by densitometry and standardized to control values (below the immunoblot). Representative of those obtained in three separate experiments. *C,* effects of roscovitine on TRAIL-induced DNA fragmentation. Cells were incubated with 10 ng/mL TRAIL with or without 25 μmol/L roscovitine for 16 hours and DNA fragmentation was quantified by PI/FACS. *Columns,* mean ($n = 3$); *bars,* ±SE.

The transcription factor NF-κB has received considerable attention for its role in cancer cell survival pathways (38). Bortezomib is a potent inhibitor of NF-κB activation via stabilization of NF-κB's physiologic inhibitor, IκBα, and its effects as a NF-κB inhibitor have been used to sensitize cancer cells to other death stimuli (38). Although inhibition of NF-κB was an attractive explanation for bortezomib's effects on TRAIL sensitivity, we were unable to mimic them with a more specific inhibitor of the pathway (the IKK inhibitor PS-1145). Rather, TRAIL sensitization was associated with the accumulation of p21 and inhibition of cdk2 activity, and it was reversed in cells transfected with an siRNA construct specific for p21. Although it is possible that p21 promotes TRAIL sensitivity via a direct mechanism, the observation that chemical cdk inhibitors like roscovitine (Fig. 3*C*; ref. 39) and flavopiridol (40–42) can also synergistically sensitize cells to TRAIL strongly suggests that p21's effects are mediated by cdk inhibition. Based on these results, we would predict that any stimulus that directly or indirectly causes cdk inhibition would sensitize cancer cells to TRAIL-mediated cell death. Support for this concept comes from the observation that tumor cells are most sensitive to TRAIL in the G_1 phase of the cell cycle (43), and DNA damaging agents synergize with TRAIL to promote apoptosis in cells that retain wild-type p53 (44), where p53-mediated p21 expression and cell cycle arrest should occur. Accumulation of p21 also underlies TRAIL sensitization induced by resveratrol (45) and probably contributes to the synergistic increases in apoptosis observed in cells treated with TRAIL plus histone deacetylase (HDAC) inhibitors (46, 47).

Although our data suggest that p21-mediated cdk inhibition is responsible for the increased caspase-8 activation observed in cells treated with bortezomib plus TRAIL, further study is required to elucidate the specific mechanisms involved. One issue is our observation that enhanced caspase-8 processing was detected as early as 30 minutes after treatment with TRAIL plus bortezomib, which was somewhat faster than the kinetics of p21 accumulation measured by immunoblotting. This observation coupled with the incomplete suppression of DNA fragmentation observed in the p21- or p27-silenced cells suggest that additional bortezomib-sensitive mechanism(s) are involved. Studies in other models concluded that bortezomib enhanced surface DR5 expression (48) and decreased levels of c-FLIP (48, 49), both of which could contribute to the increased caspase-8 activation observed. We have confirmed that bortezomib and roscovitine increase surface DR5 expression in the LNCaP-Pro5 and 253J B-V cells, but their effects are delayed (>12 hours) relative to the rapid kinetics of caspase activation and DNA fragmentation (4-8 hours).[3] We also investigated the effects of bortezomib on expression of c-FLIP and the TRAIL decoy receptors (DcR-1 and DcR-2) in our cells and did not detect any obvious changes that might account for the phenomenon.[4] On the other hand, the FADD adaptor protein is known to be phosphorylated in a cell cycle–sensitive manner (50–52), and in preliminary studies we have found that combined treatment with bortezomib plus TRAIL leads to changes in FADD phosphorylation

[3] L. Lashinger and M. Shrader, unpublished observations.
[4] L. Lashinger, unpublished observations.

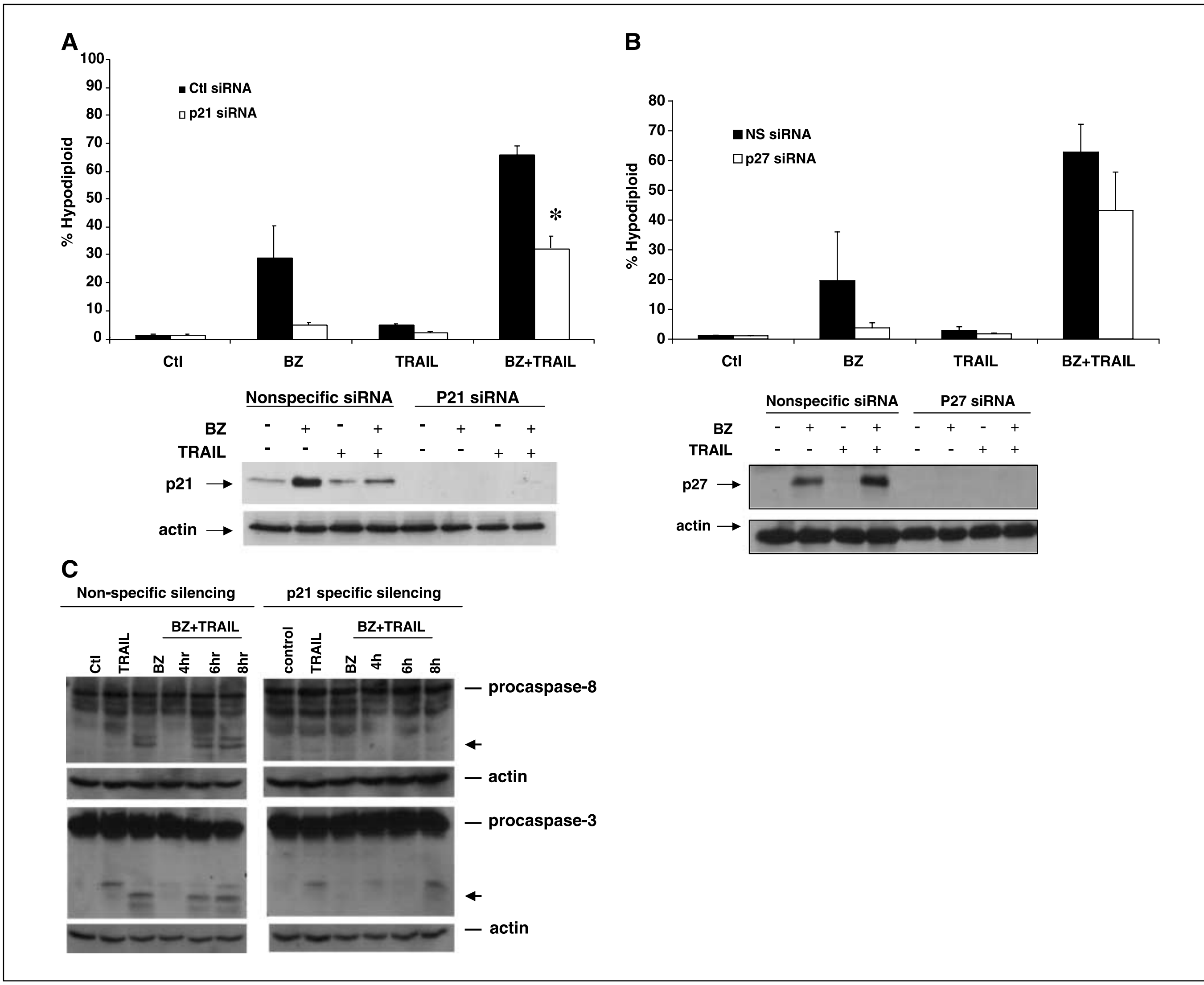

Figure 4. Accumulation of p21 is required for bortezomib-mediated TRAIL sensitization. *A,* effects of p21 silencing on DNA fragmentation. Cells were transiently transfected with an siRNA construct specific for p21 or a nonspecific control construct for 48 hours. Cells were incubated for 8 hours with 100 nmol/L bortezomib, 10 ng/mL TRAIL, or both agents, and DNA fragmentation was measured by PI/FACS. *Columns,* mean (n = 3); *bars,* ±SE. *P < 0.01. *Bottom,* effects of p21 silencing on protein expression were measured by immunoblotting. Representative of those obtained in three separate experiments. *B,* effects of p27 silencing on DNA fragmentation. Cells were transiently transfected with an siRNA construct specific for p27 or a nonspecific control construct for 48 hours. Cells were then incubated for 8 hours with 100 nmol/L bortezomib, 10 ng/mL TRAIL, or both agents, and DNA fragmentation was measured by PI/FACS. *Columns,* mean (n = 3); *bars,* ±SE. *Bottom,* effects of p27 silencing on protein expression were measured by immunoblotting. Representative of those obtained in three separate experiments. *C,* effects of p21 silencing on procaspase-8 and procaspase-3 activation. Cells were transfected with the p21-specific siRNA construct or a control construct for 48 hours. Cells were incubated for 8 hours with 100 nmol/L bortezomib, 10 ng/mL TRAIL, or both agents for 8 hours, and procaspase-8 and procaspase-3 were visualized by immunoblotting. Actin served as a control for protein loading. *Arrows,* mature large subunits of caspase-8 and caspase-3. Note that these mature forms are absent in the p21-silenced cells.

that are not observed in response to treatment with either agent alone.[5] Therefore, in ongoing studies, we are attempting to define the potential biological significance of these changes in FADD phosphorylation in our cell lines.

Accumulating evidence indicates that cell cycle progression and cell death are mechanistically interrelated (53). Specifically, alterations that promote cell cycle progression often sensitize cells to death, whereas processes that inhibit cell cycle progression block cell death (53). Most of the investigational agents being studied at present (i.e., growth factor receptor antagonists, kinase inhibitors, HDAC inhibitors, bortezomib, etc) arrest cells in G_1 (54), which may enable them to reinforce the growth inhibitory/cytostatic effects of conventional chemotherapy but probably does not make them particularly effective in promoting cell killing. Coupled with the other studies described above, our data strongly suggest that cell cycle arrest at the G_1-S checkpoint promotes sensitivity to TRAIL-mediated apoptosis in cancer cells, which places it in a unique category relative to other death-inducing stimuli. Thus, TRAIL-based combination therapy seems qualitatively different from other combinations of biological and cytotoxic agents because it is most active in cells

[5] S. Williams, unpublished observations.

that have been growth arrested. The data provide a compelling rationale for doing more extensive studies to optimize the antitumor activities of these combinations in appropriate preclinical models in preparation for clinical studies in patients. Our preliminary studies[4] indicate that biologically active doses of bortezomib and recombinant human TRAIL can be delivered to nude mice without generating systemic toxicity.

Acknowledgments

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References

1. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995;3:673–82.
2. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157–63.
3. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–62.
4. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997;277:818–21.
5. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science 1997;276:111–3.
6. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 1997;277:815–8.
7. Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N, Tschopp J. Characterization of two receptors for TRAIL. FEBS Lett 1997;416:329–34.
8. Kischkel FC, Hellbardt S, Behrmann I, et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J 1995;14:5579–88.
9. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003;22:8628–33.
10. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 2000;12:611–20.
11. Sprick MR, Weigand MA, Rieser E, et al. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 2000;12:599–609.
12. Bodmer JL, Holler N, Reynard S, et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol 2000;2:241–3.
13. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 2004;4:333–9.
14. Pickart CM. Back to the future with ubiquitin. Cell 2004;116:181–90.
15. Goldberg AL, Stein R, Adams J. New insights into proteasome function: from archaebacteria to drug development. Chem Biol 1995;2:503–8.
16. Rock KL, Gramm C, Rothstein L, et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 1994;78:761–71.
17. Adams J, Palombella VJ, Elliott PJ. Proteasome inhibition: a new strategy in cancer treatment. Invest New Drugs 2000;18:109–21.
18. Adams J. Proteasome inhibition in cancer: development of PS-341. Semin Oncol 2001;28:613–9.
19. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999;59:2615–22.
20. Shah SA, Potter MW, McDade TP, et al. 26S proteasome inhibition induces apoptosis and limits growth of human pancreatic cancer. J Cell Biochem 2001;82:110–22.
21. Bold RJ, Virudachalam S, McConkey DJ. Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. J Surg Res 2001;100:11–7.
22. Sunwoo JB, Chen Z, Dong G, et al. Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-κB, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. Clin Cancer Res 2001;7:1419–28.
23. Nawrocki ST, Sweeney-Gotsch B, Takamori R, McConkey DJ. The proteasome inhibitor bortezomib enhances the activity of docetaxel in orthotopic human pancreatic tumor xenografts. Mol Cancer Ther 2004;3:59–70.
24. Pettaway CA, Pathak S, Greene G, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Cancer Res 1996;2:1627–36.
25. Dinney CP, Fishbeck R, Singh RK, et al. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. J Urol 1995;154:1532–8.
26. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–9.
27. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84:1415–20.
28. Jeremias I, Kupatt C, Baumann B, Herr I, Wirth T, Debatin KM. Inhibition of nuclear factor κB activation attenuates apoptosis resistance in lymphoid cells. Blood 1998;91:4624–31.
29. Keane MM, Rubinstein Y, Cuello M, et al. Inhibition of NF-κB activity enhances TRAIL mediated apoptosis in breast cancer cell lines. Breast Cancer Res Treat 2000;64:211–9.
30. Nawrocki ST, Bruns CJ, Harbison MT, et al. Effects of the proteasome inhibitor PS-341 on apoptosis and angiogenesis in orthotopic human pancreatic tumor xenografts. Mol Cancer Ther 2002;1:1243–53.
31. Hideshima T, Chauhan D, Richardson P, et al. NF-κB as a therapeutic target in multiple myeloma. J Biol Chem 2002;277:16639–47.
32. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999;11:255–60.
33. Kamat AM, Karashima T, Davis DW, et al. The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors *in vivo*. Mol Cancer Ther 2004;3:279–90.
34. Williams S, Pettaway C, Song R, Papandreou C, Logothetis C, McConkey DJ. Differential effects of the proteasome inhibitor bortezomib on apoptosis and angiogenesis in human prostate tumor xenografts. Mol Cancer Ther 2003;2:835–43.
35. An WG, Hwang SG, Trepel JB, Blagosklonny MV. Protease inhibitor-induced apoptosis: accumulation of wt p53, p21WAF1/CIP1, and induction of apoptosis are independent markers of proteasome inhibition. Leukemia 2000;14:1276–83.
36. Blagosklonny MV. Flavopiridol, an inhibitor of transcription: implications, problems and solutions. Cell Cycle 2004;3:1537–42.
37. Demidenko ZN, Blagosklonny MV. Flavopiridol induces p53 via initial inhibition of Mdm2 and p21 and, independently of p53, sensitizes apoptosis-reluctant cells to tumor necrosis factor. Cancer Res 2004;64:3653–60.
38. Orlowski RZ, Baldwin AS Jr. NF-κB as a therapeutic target in cancer. Trends Mol Med 2002;8:385–9.
39. Kim EH, Kim SU, Shin DY, Choi KS. Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. Oncogene 2004;23:446–56.
40. Kim DM, Koo SY, Jeon K, et al. Rapid induction of apoptosis by combination of flavopiridol and tumor necrosis factor (TNF)-α or TNF-related apoptosis-inducing ligand in human cancer cell lines. Cancer Res 2003;63:621–6.
41. Taniai M, Grambihler A, Higuchi H, et al. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. Cancer Res 2004;64:3517–24.
42. Rosato RR, Dai Y, Almenara JA, Maggio SC, Grant S. Potent antileukemic interactions between flavopiridol and TRAIL/Apo2L involve flavopiridol-mediated XIAP downregulation. Leukemia 2004;18:1780–8.
43. Jin Z, Dicker DT, El-Deiry WS. Enhanced sensitivity of G_1 arrested human cancer cells suggests a novel therapeutic strategy using a combination of simvastatin and TRAIL. Cell Cycle 2002;1:82–9.
44. Wang S, El-Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. Proc Natl Acad Sci U S A 2003;100:15095–100.
45. Fulda S, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. Cancer Res 2004;64:337–46.
46. Rosato RR, Almenara JA, Dai Y, Grant S. Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells. Mol Cancer Ther 2003;2:1273–84.
47. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene 2004;23:6261–71.
48. Johnson TR, Stone K, Nikrad M, et al. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. Oncogene 2003;22:4953–63.
49. Sayers TJ, Brooks AD, Koh CY, et al. The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. Blood 2003;102:303–10.
50. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Kishi M, Konishi N. Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis. Carcinogenesis 2004;25:1089–97.
51. Scaffidi C, Volkland J, Blomberg I, Hoffmann I, Krammer PH, Peter ME. Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. J Immunol 2000;164:1236–42.
52. Alappat EC, Volkland J, Peter ME. Cell cycle effects by C-FADD depend on its C-terminal phosphorylation site. J Biol Chem 2003;278:41585–8.
53. Harrington EA, Fanidi A, Evan GI. Oncogenes and cell death. Curr Opin Genet Dev 1994;4:120–9.
54. Owa T, Yoshino H, Yoshimatsu K, Nagasu T. Cell cycle regulation in the G_1 phase: a promising target for the development of new chemotherapeutic anticancer agents. Curr Med Chem 2001;8:1487–503.